

Short sequence-paper

Cloning of a sugar utilization gene cluster in *Aspergillus parasiticus*

Jiujiang Yu *, Perng-Kuang Chang, Deepak Bhatnagar, Thomas E. Cleveland

USDA/ARS, Southern Regional Research Center, 1100 Robert E. Lee Blvd., New Orleans, LA 70179, USA

Received 22 March 2000; received in revised form 17 May 2000; accepted 24 May 2000

Abstract

At one end of the 70 kb aflatoxin biosynthetic pathway gene cluster in *Aspergillus parasiticus* and *Aspergillus flavus* reported earlier, we have cloned a group of four genes that constitute a well-defined gene cluster related to sugar utilization in *A. parasiticus*: (1) *sugR*, (2) *hxtA*, (3) *glcA* and (4) *nadA*. No similar well-defined sugar gene cluster has been reported so far in any other related *Aspergillus* species such as *A. flavus*, *A. nidulans*, *A. sojae*, *A. niger*, *A. oryzae* and *A. fumigatus*. The expression of the *hxtA* gene, encoding a hexose transporter protein, was found to be concurrent with the aflatoxin pathway cluster genes, in aflatoxin-conducive medium. This is significant since a close linkage between the two gene clusters could potentially explain the induction of aflatoxin biosynthesis by simple sugars such as glucose or sucrose. © 2000 Published by Elsevier Science B.V.

Keywords: Genome structure; Sugar regulation; Zinc-finger; Aflatoxin; Hexose transport; Glucosidase; NADH oxidase

Contamination of agricultural commodities with aflatoxins are of serious health and economic concerns worldwide [1]. The discovery of the aflatoxin pathway gene cluster led to a rapid identification and cloning of almost all the genes involved in aflatoxin biosynthesis [2–4] including the positive pathway regulator, *afIR*. It is known that carbon source plays a vital role in the regulation of aflatoxin production [5–7]. *Aspergillus parasiticus* mycelia grown in the medium containing the simple sugars such as glucose, sucrose, maltose and galactose support aflatoxin production, while lipids and starch do not support aflatoxin production. Some peptides such as peptone even inhibit aflatoxin production by either directly or indirectly affecting aflatoxin pathway gene transcription [3,5,8,9]. In *Saccharomyces cerevisiae*, over a dozen high- and low-affinity hexose transporter genes have been identified [10–13]. The *CASUC1* (accession number S75352) gene in *Candida albicans* was identified to affect sucrose utilization and α -glucosidase activity [14]. *CASUC1* gene encodes a zinc-finger containing protein with 28% identity to that of the maltose regulatory gene (*MAL63*, accession number M36537) of *S. cerevisiae*. Recently, a cluster of three maltose utilization genes, *MAL23* (accession number AF002704), *MAL43* (accession number M81157), *MAL63* [15], was identified and may be involved in mal-

tose uptake. In the non-aflatoxigenic strain *A. oryzae*, a cluster of two genes, *agdA* (accession number D45179), encoding an α -glucosidase, and *amyR* (GenBank accession number AB012945), encoding the *agdA* transcription activator, has been cloned, while its position relative to the aflatoxin pathway genes is unclear. However, no sugar utilization-related gene cluster has ever been identified from the aflatoxigenic fungal strains *A. parasiticus* and *A. flavus* even though sugars have a marked effect on toxin synthesis. We report here the identification and cloning of a well-defined cluster of four genes which share homologies with some of the above mentioned sugar utilization genes.

Identification and organization of the sugar utilization gene cluster in the *A. parasiticus* genome. Following the cloning of the 70 kb aflatoxin pathway gene cluster [2], we identified the genes *cypX* and *moxY* (Fig. 1, GenBank accession number AF169016) to be the end of the aflatoxin pathway gene cluster in one orientation [16]. Following a 5 kb non-coding region (with no identified open reading frame, ORF) downstream of the *moxY* gene, four new transcripts have been identified from aflatoxigenic *A. parasiticus* wild-type strain SRRC 143 (ATCC 56775) (Fig. 1). On the other boundary of these four genes, a 4 kb DNA sequence was identified with no ORF. These four genes are linked together to form a sugar utilization gene cluster in *A. parasiticus*: (a) *nadA* gene which has homology to genes encoding a NADH oxidase; (b) *hxtA* gene which has homology to genes encoding a hexose transporter pro-

* Corresponding author. Fax: +1-504-286-4419;
E-mail: jiuju@commsvr.srrc.usda.gov

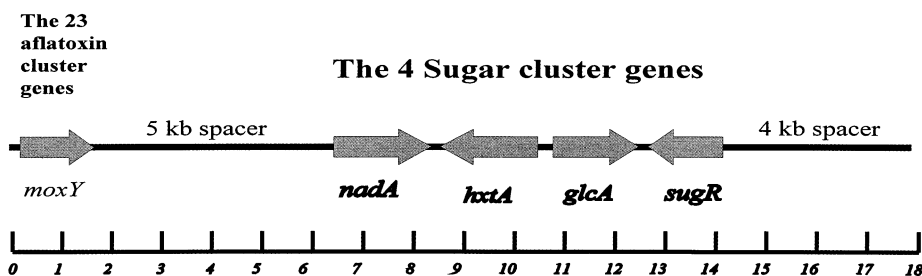


Fig. 1. Schematic presentation of sugar utilization gene cluster in *A. parasiticus* SRRC 143. Arrows show the genes and their directions of transcription. The *moxY* gene shown on the scheme is at one end of the 23 aflatoxin pathway cluster genes. The distances between the two gene clusters and between individual genes in kb are also shown.

tein; (c) *glcA* gene which has homology to genes encoding α -1,4- or α -1,6-glucosidases; (d) *sugR* gene which has homology to Cys-6-type transcription factor genes encoding sugar utilization regulatory proteins. The genomic DNA and cDNA sequences of these four genes have been determined (GenBank accession number AF168613) and the putative functions of these four genes have been proposed based on sequence homology. The unique location of the two clusters in the fungal genome makes it relevant to study the relationship of regulation of sugar utilization and its effect on the regulation of aflatoxin formation. It could be a step towards understanding the complex regulation of aflatoxin biosynthesis, which is dependent on several factors such as carbon source, nitrogen source, temperature and pH [7,17].

The *hxtA* gene encodes a hexose transporter protein for hexose uptake. The *hxtA* gene coding sequence consists of 1542 nucleotides capable of encoding 513 amino acids with a protein calculated molecular mass of 56 kDa (Fig. 2). There are two intervening sequences of 102 bp

and 57 bp, respectively (Fig. 2). Sequence comparison results showed that the *hxtA* gene product has significant overall sequence homologies (25–30% identity and 45–50% positive) to over a dozen yeast hexose transport proteins [12,18] including both high- and low-affinity glucose transporters. Several GC boxes were identified in the promoter region of the *hxtA* gene. The GC boxes may be the target binding sites by a negative regulator such as *Mig1* in the yeast system to repress the transcription of hexose transporters [13].

The hydropathy plot of *A. parasiticus* *hxtA* gene product demonstrated that the amino acid sequence shows a high degree of hydrophobicity and contains 12 putative membrane spanning segments (Fig. 2), which is consistent with all other hexose transporter proteins discovered so far [13]. The transmembrane protein could presumably regulate the transport of sugar molecules across a membrane. In *A. parasiticus*, there may exist several hexose transport genes, other than *hxtA*, that produce functionally similar hexose transporters for various functions in the cell requir-

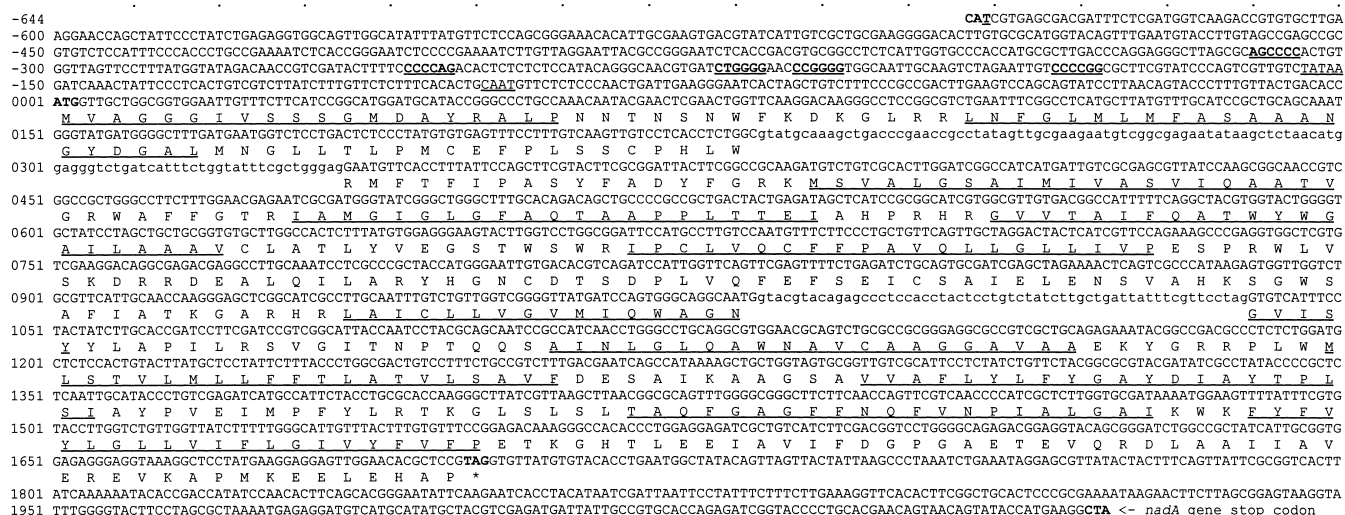


Fig. 2. The genomic DNA sequences and deduced amino acid sequences of *hxtA*. The numbers refer to nucleotides beginning from translation initiation codon ATG. The translation start codon (ATG) and the translation termination codon are printed in bold face letters. The intron sequences are denoted by lower case letters. The deduced amino acid sequence is presented under each corresponding codon and the translation termination is labeled by a star (*). In the promoter region, the putative GC boxes (CCGGGG or GGCCCC) for *Mig1* repressor binding [13] in yeast, a putative CAAT box, and the putative transmembrane segments are underlined. The GenBank accession number of the four sugar utilization genes, *sugR*, *hxtA*, *glcA* and *nadA*, including the 4 kb non-coding region from *A. parasiticus* is AF168613, and that of the DNA sequence of the 5 kb spacer region between the aflatoxin pathway and sugar utilization gene clusters including the last aflatoxin pathway gene *moxY* (Fig. 1) is AF169016.

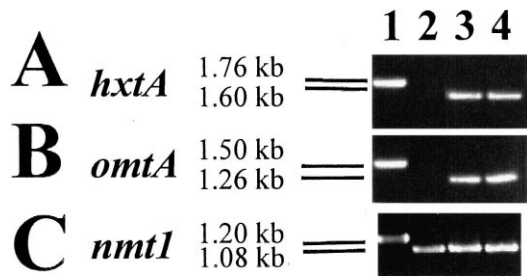


Fig. 3. Transcript detection of the *hxtA* gene by RT-PCR in *A. parasiticus*. RT-PCR was performed to detect mRNAs from GMS and PMS media in *A. parasiticus* for *hxtA* (A), *omtA* (B) and *nmt1* (C). Aflatoxinogenic fungal strain *A. parasiticus* SRRC 143 was grown in PMS (non-aflatoxin-conductive) medium for 48 h and shifted to GMS (aflatoxin-conductive) medium at 30°C with constant shaking at 150 rpm. Total RNA was isolated using RNeasy Total RNA kit (Qiagen Inc., Valencia, CA, USA) from the mycelia harvested at 0, 24 and 48 h after growing in the GMS medium and mRNA was purified subsequently using the PolyATract mRNA isolation system (Promega Corp., Madison, WI, USA). First strand cDNA was synthesized by Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) and used as the template in RT-PCR. The templates used in lane 1, genomic DNA; lane 2, RNAs purified from mycelia grown in PMS medium for 48 h; lane 3, RNAs purified from mycelia grown in GMS medium for 24 h; lane 4, RNAs purified from mycelia grown in GMS medium for 48 h. RT-PCR was performed by denaturing at 94°C for 15 s, annealing at 56°C for 30 s, extension at 72°C for 2 min, 30 cycles with a final extension at 72°C for 5 min. PCR products were separated in 2% agarose gel under 80 V for 90 min.

ing the transport of various hexoses, such as glucose or galactose. A gene, *hxt1*, potentially encoding a hexose transporter, was previously cloned (GenBank accession number AF010145). Sequence comparison of *hxtA* cloned in this study and *hxt1* cloned earlier failed to identify significant homology at the DNA level. However, the homology at the amino acid level demonstrated 23% identity and 41% positive. Both *hxtA* and *hxt1* gene products contain 12 membrane spanning segments, a typical feature for all of the hexose transporters. The chromosome location of the *hxt1* gene and its association with other genes is not clear.

The *hxtA* gene is expressed concurrently with the aflatoxin pathway genes. The expression of the four cloned genes (*hxtA*, *sugR*, *glcA* and *nadA*) in aflatoxin-conductive (GMS for glucose mineral salt, [5]) and non-conductive (PMS for peptone mineral salt, [5]) media was studied by reverse

transcription (RT)-PCR experiments. The *hxtA* gene was demonstrated to be expressed in GMS but not PMS media (Fig. 3A). This result was confirmed by Northern blot analysis (data not shown). Preliminary results by RT-PCR experiments demonstrated that the *sugR*, *glcA* and *nadA* genes were expressed in both GMS and PMS media (i.e. constitutively expressed, data not shown). RT-PCR using primers internal to the *hxtA* coding sequence resulted in a 1.60 kb fragment from poly-A mRNA isolated from 24 h and 48 h mycelia grown in GMS medium (Fig. 3A, lanes 3 and 4), but not from mycelia grown on non-aflatoxin-conductive PMS medium (Fig. 3A, lane 2). This gene expression pattern is consistent with that of the aflatoxin pathway cluster genes such as *omtA* (Fig. 3B). Using the constitutively expressed (independent of sugar source) *nmt1* gene as a positive control, a 1.08 kb RT-PCR product was amplified from both GMS and PMS media (Fig. 3C).

The *sugR* gene encodes a regulatory protein containing a typical zinc-finger motif. The *sugR* gene coding sequence consists of 1476 nucleotides and is capable of encoding a protein of 491 amino acids with a calculated molecular mass of 54.9 kDa. There is only one intervening sequence of 46 bp located close to the 3' end of the *sugR* coding region (sequence not shown, AF168613). Blast search for possible homologies in the GenBank database showed that *sugR* gene product in the region from amino acid residue 233 to 425 has significant homologies to the sugar regulatory proteins in other organisms: its amino acid sequence showed 25% identity and 47% similarity to that of *CASUC1* in *C. albicans* [14]; 30% identity and 47% similarity to the protein encoded by *amyR* in *A. oryzae*. More importantly, the most common feature among sugar regulatory proteins is the presence of the typical C6 zinc-finger motif close to the N-terminal end for DNA binding (Fig. 4). The spaces between Cys-4 and Cys-5 consist of five amino acids for the sugar cluster gene activators (CX₂CX₆CX₅CX₂CX₆C) as compared to the six amino acids found in aflatoxin pathway regulator protein AFLR (encoded by *aflR*) and *GAL4*-type (CX₂CX₆CX₆CX₂CX₆C) transcription regulating proteins.

The *glcA* gene encodes a glucosidase. The coding sequence of the *glcA* gene consists of 1707 nucleotides and

consensus:	Position	C x x C x x x K x R C x x x x P - C x x C x x x x x C
<i>sugR</i> (A.p.):	(7-33)	M V R R R A <u>C D G C S L R K T R C S G G O P - C O P C A O S G F E C</u> S Y L K P A A K
<i>CASUC1</i> (C.a.):	(13-39)	A P Y T R P <u>C D S C S F R K V K C D M K T P - C S R C V L N N L K C</u> T N N R I R K K
<i>MAL63</i> (S.c.):	(8-34)	M G I A K Q S <u>C D C C R V R V R C D R N K P - C N R C I O R N L N C</u> T Y L Q P L K K
<i>amyR</i> (A.o.):	(28-54)	E K P P K Q A <u>C D N C R R R K I K C S R E L P - C D K C O R L L L S C</u> S Y S D V L R R
<i>aflR</i> (A.p.):	(29-56)	A R K L R D S <u>C T S C A S S K V R C T K E K P A C A R C I E R G L A C</u> Q Y M V S K R M
<i>GAL4</i> (S.c.):	(11-38)	L S S I E Q A <u>C D I C R L K K L K C S K E K P K C A K C L K N N W E C</u> R Y S P K T K R

Fig. 4. The comparisons of the C6 zinc-finger motifs of the sugar regulatory gene (*sugR*) and those from other organisms. Comparisons of the C6 zinc-finger proteins involved in the sugar utilization gene activation from *A. parasiticus* (*sugR*, AF168613), *C. albicans* (*CASUC1*, S75352), *S. cerevisiae* (*MAL63*, M36537), *A. oryzae* (*amyR*, AB012945) are shown. The aflatoxin pathway gene regulator (*aflR*, L22177) and galactose gene activator (*GAL4*, accession number Z73604) are also included for comparison. The zinc-finger motifs are underlined and the six cysteine residues are printed in bold face letters. The position of the zinc-finger from translation start site are labeled.

is capable of encoding 568 amino acids with a protein molecular mass of 64.9 kDa. There are three intervening sequences of 76 bp, 58 bp and 49 bp, respectively (sequence not shown, AF168613). Search for possible homologies in the GenBank database showed that *glcA* gene product has significant homologies (40–50% identity and 60–65% positive at amino acid level) to other glucosidase proteins such as α -1,6-glucosidase, α -1,4-glucosidase/maltase, sucrose-isomaltase in yeast and *Bacillus* species. The glucosidase is an enzyme that is responsible for the release of glucose from starch or oligosaccharides.

The *nadA* gene encodes a NADH oxidase. This gene showed significant amino acid sequence homology to NADH oxidase in *C. elegans* (40% at the amino acid level), *Bacillus subtilis* and other organisms. The coding sequence of the *nadA* gene consists of 1335 nucleotides and is capable of encoding 444 amino acids with a protein molecular mass of 48.5 kDa. There is a single intron sequence of 61 bp (sequence not shown, AF168613). The Blast search in the GenBank database showed that the *nadA* gene product has significant homology (30–45% identity and 50–56% positive at the amino acid level) to other NADH oxidases.

After hydrolysis of glycogen or starch to glucose, in glycolysis, one molecule of C6 glucose is further broken down into pyruvate. NAD⁺ and NADH are required for internal coupling in glycolysis under anaerobic conditions, or the tricarboxylic acid cycle under aerobic conditions. NADH oxidase is probably involved in re-oxidation of NADH to NAD⁺ for the constant replenishment of NAD⁺. The NADH might be generated by dehydrogenases but also by other enzymes or pathways.

References

- [1] C.F. Jelinek, A.E. Pohland, G.E. Wood, J. Assoc. Off. Anal. Chem. 72 (1989) 223–230.
- [2] J. Yu, P.-K. Chang, J.W. Cary, M. Wright, D. Bhatnagar, T.E. Cleveland, G.A. Payne, J.E. Linz, Appl. Environ. Microbiol. 61 (1995) 2365–2371.
- [3] T.E. Cleveland, J.W. Cary, R.L. Brown, D. Bhatnagar, J. Yu, P.-K. Chang, C.A. Chlan, K. Rajasekaran, Bull. Inst. Compr. Agr. Sci. Kinki Univ. 5 (1997) 75–90.
- [4] C.P. Woloshuk, R. Prieto, FEMS Microbiol. Lett. 160 (1998) 169–176.
- [5] J. Adye, R.I. Mateles, Biochim. Biophys. Acta 86 (1964) 418–420.
- [6] T.A. Venkatasubramanian, in: J.V. Rodricks, C.W. Hesseltine and M.A. Mehlman (Eds.), Mycotoxins in Human and Animal Health, Pathotox Publishers, Inc., IL, 1977, pp. 83–98.
- [7] G.A. Payne, M.P. Brown, Annu. Rev. Phytopathol. 36 (1998) 329–362.
- [8] D. Bhatnagar, K.C. Ehrlich and T.E. Cleveland, in: D. Bhatnagar, E.B. Lillehoj and D.K. Arora (Eds.), Handbook of Applied Mycology, Vol. 5, Mycotoxins in ecological systems, Marcel Dekker, Inc., New York, 1992, pp. 255–286.
- [9] J. Yu, P.-K. Chang, K.C. Ehrlich, J.W. Cary, B.G. Montalbano, J.M. Dyer, D. Bhatnagar, T.E. Cleveland, Appl. Environ. Microbiol. 64 (1998) 4834–4841.
- [10] A. Kruckeberg, L.F. Bisson, Mol. Cell. Biol. 10 (1990) 5903–5913.
- [11] D.A. Lewis, L.F. Bisson, Mol. Cell. Biol. 11 (1991) 3804–3813.
- [12] C.H. Ko, H. Liang, R.F. Gaber, Mol. Cell. Biol. 13 (1993) 638–648.
- [13] M. Johnston, Trends Genet. 15 (1999) 29–33.
- [14] R. Kelly, K.J. Kwon-Chung, J. Bacteriol. 174 (1992) 222–232.
- [15] A.W. Gibson, L.A. Wojciechowicz, S.E. Danzi, B. Zhang, J.H. Kim, Z. Hu, C.A. Michels, Genetics 146 (1997) 1287–1298.
- [16] J. Yu, P.-K. Chang, D. Bhatnagar, T.E. Cleveland, Appl. Microbiol. Biotechnol. 53 (2000) 583–590.
- [17] C.P. Woloshuk, J.R. Cavaletto, T.E. Cleveland, Phytopathology 87 (1997) 164–169.
- [18] R.K. Freidel, M. Cirlacy, Mol. Microbiol. 15 (1995) 157–167.